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Biodegradable HEMA-based hydrogels with enhanced mechanical properties

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Abstract: Hydrogels are widely used in the biomedical field. Their main purposes are either to deliver biological active agents or to temporarily fill a defect until they degrade and are followed by new host tissue formation. However, for this latter application, biodegradable hydrogels are usually not capable to sustain any significant load. The development of biodegradable hydrogels presenting load-bearing capabilities would open new possibilities to utilize this class of material in the biomedical field. In this work, an original formulation of biodegradable photo-crosslinked hydrogels based on hydroxyethyl methacrylate (HEMA) is presented. The hydrogels consist of short-length poly(2-hydroxyethyl methacrylate) (PHEMA) chains in a star shape structure, obtained by introducing a tetra-functional chain transfer agent in the backbone of the hydrogels. They are cross-linked with a biodegradable N,O-dimethacryloyl hydroxylamine (DMHA) molecule sensitive to hydrolytic cleavage. We characterized the degradation properties of these hydrogels submitted to mechanical loadings. We showed that the developed hydrogels undergo long-term degradation and specially meet the two essential requirements of a biodegradable hydrogel suitable for load bearing applications: enhanced mechanical properties and low molecular weight degradation products. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 104B: 1161–1169, 2016.

Key Words: HEMA-based hydrogels, biodegradable hydrogels, mechanical tests

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INTRODUCTION

Hydrogels are widely used in biomedical applications either as vehicles to deliver a biological agent or as scaffolds to fill a defect. In particular, the biodegradable version of hydrogels is of great interest since upon their degradation, formation of neo-tissues can be obtained in the defect.[1–4] However, due to the weak mechanical properties of biodegradable hydrogels, their application is very limited in tissues functioning under load situations.[5–8] Therefore, there is a clear need for developing biodegradable hydrogels with an enhanced load-bearing capability.

Biodegradable hydrogels may be produced based on the cleavage of hydrolytically or enzymatically labile bonds[5–8] or dissolution of physical cross-links formed via hydrophobic, electrostatic, or hydrogen-bonding forces.[9,10] Due to these weak cross-linking mechanisms, most of the developed biodegradable hydrogels exhibit poor mechanical properties.[11,12] In contrast, synthetic chemically cross-linked hydrogels with strong covalent bonds present an enhanced mechanical strength.[11,13,14] However, the strong covalent bonds limit the degradation of these hydrogels.[11,14]

In order to develop a hydrogel with simultaneously strong mechanical properties and degradation capabilities, we focused our attention on methacrylate polymers such as poly(2-hydroxyethyl methacrylate) (PHEMA). Hydrogels made of these polymers were shown to be biocompatible, to have tunable mechanical properties, and they can be fabricated in different architectures.[15–17] We recently showed that HEMA-based hydrogels present very high load tolerance and especially resistance to crack propagation when we increased their dissipation properties.[18] However, because of its high biostability,[16] PHEMA has not been successfully used as a biodegradable hydrogel yet, despite several studies have focused on this aspect.[6,17,19–22] Although some of the proposed structures presented degradation capabilities to some extent, the resulting hydrogels had two major deficiencies. First, as expected they showed very poor mechanical properties due to the structure of the cross-linker used[11,22] or to the mechanism of cross-linking.[23,24] Second, their degradation products consisted of very long PHEMA chains.[6] Indeed, the structure of HEMA-based hydrogels is composed of long PHEMA chains interconnected with cross-linker molecules. It has been reported that under physiological conditions, PHEMA can be hydrolyzed to poly(methyl methacrylate).[25] However, if the degradation occurs just via dissolution of the cross-linkers, the resulting degradation product consists of very long chains of coiled PHEMA fragments, which cannot be cleared out of the body.[6,26,27]

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To overcome these two drawbacks, we developed an original formulation of biodegradable HEMA-based hydrogels, which present enhanced mechanical properties and degradation products with low molecular weight. These hydrogels were developed based on HEMA cross-linked with a short hydrolysable molecule N,O-dimethacryloyl hydroxylamine (DMHA) and employing a tetra-functional chain transfer agent in the backbone of these hydrogels. DMHA provides high initial mechanical properties and the tetra-functional molecule breaks down the PHEMA long backbone chains to small star-like molecules.

MATERIALS AND METHODS

Materials

All materials were purchased from Aldrich (Buchs, Switzerland) and were stored at 4°C until use unless otherwise specified. Hydroxylamine hydrochloride, methacryloyl chloride (−20°C), pyridine, chloroform, and hydrochloric acid were all used for synthesizing N,O-dimethacryloyl hydroxylamine. 2–2-hydroxyethyl methacrylate (HEMA, 97%) was purified using basic aluminum oxide column chromatography to remove inhibitors. 2,2-dimethoxy-2-phenylacetophenone (DPAP) (Irgacure-651, 99%) was used as photo initiator and prepared as an ethanolic solution of DPAP (57 mg/mL solution, each ml = 0.2 m*M*). Pentaerythritol tetrakis(3-mercaptopropionate) was used as received without further purification.

Cell culture media contained 10 mL Dulbecco's Modified Eagle Medium (DMEM) with 25 m*M* dextrose and 1 mM sodium pyruvate (Life Technologies Ltd, Paisley, UK), 5.97 m*M* L-Glutamine (Life Technologies Ltd), 10% fetal bovine serum (Sigma, St. Louis, MO). Giemsa's azur eosin methylene blue solution for microscopy (Merck, Darmstadt, Germany) and CellTiter (G3580 Promega, Fitchburg, WI) were used for the cell study.

DMHA synthesis

N,O-Dimethacryloyl hydroxylamine (DMHA) was synthesized following a slightly modified protocol initially proposed by South et al.[28] Ten grams of hydroxylamine hydrochloride (0.145 mol) was added in a 500 mL round-bottom flask and put under N_2 gas for ~30 min to remove moisture. Totally, 50 mL pyridine was added by syringe and stirred with the hydroxylamine hydrochloride until complete dissolution. The solution was maintained under N_2 and in an ice bath to keep the temperature of the reaction mixture below 20°C. Methacryloyl chloride, 29 mL (0.3 mol), was added dropwise. We reduced the system's exposure to light as much as possible by covering the flask with an aluminum foil. The reaction was then stirred for an additional 5 h. After complete conversion, 100 mL of chloroform was added, and the solution became a transparent yellow-brown liquid. Afterward, 100 mL of hydrochloric acid (1.5 molar) was added dropwise, while the solution was maintained in the ice bath and under aluminum foil. The mixture turned turbid. The solution was poured into a separatory funnel and was washed with 100 mL of deionized water until the aqueous layer became clear (four washes). The organic layer was then dried over magnesium sulfate and filtered. We removed chloroform by rotary evaporation in a dark 200 mL round bottom flask and then, the yellow oily product was dried overnight on a Schlenk line. The product was a yellow highly viscous liquid. Experimental yield was approximately 30%. The chemical composition of DMHA was verified with ^{1}H NMR using a Varian Unity 300 MHz NMR spectrometer (Fitchburg, MI). For ^{1}H NMR test, sample (10 μL) was dissolved in DMSO-d_6. The following chemical shifts (δ) were observed in NMR signal: 1.83 [s, 3H, N-CO-C(CH_3)=CH_2], 1.97 [s, 3H, —CO—C(CH_3) =CH_2], 5.48, 5.73 [s, 2H, —N—CO—C(CH_3) =CH_2], 5.85, 6.28 [s, 2H, —O—CO—C(CH_3) =CH_2]

TABLE I. Hydrogel Composition

Sample Name	HEMA (% mol)	DMHA (% mol)	Tetrakis (% mol)	Water (% Total Volume)
S1	100	10	0	40
S2	100	10	0.25	40
S3	100	1	0	65
S4	100	1	0.25	65

Hydrogels synthesis

PHEMA hydrogels were prepared with N,O-dimethacryloyl hydroxylamine (DMHA) as cross-linker (1% and 10% of mol) and water (65% and 40% of total mixture volume). In another group of hydrogels we added a four functional group molecule, pentaerythritol tetrakis(3-mercaptopropionate) (0.25% of mol). The term "Tetrakis" will be used for this material in the following sections. Material compositions for all four groups of samples are summarized in Table I (S1, S2, S3, and S4). The mixture containing the photo initiator DPAP (0.1% of mol) was stirred and sonicated for 1 min. It was then transferred to cylindrical wells (6 mm diameter and 3 mm depth), placed under UV lamp (365 nm, 8 watt) (Upland, CA) positioned 10 cm from the samples and irradiated for 15 min. The system was maintained below 25°C during polymerization by air circulation. Hydrogels were then carefully removed from the wells, washed to remove unreacted materials, and immersed in water for 1 week. Figure 1 shows the expected structure of the hydrogels with and without Tetrakis. In the presence of Tetrakis, hydrogels present a star structure in which four short PHEMA chains are linked by ester bonds to a tetra-functional Tetrakis molecule. The branches of HEMA in the ideal case when monomer conversion reaches 100% contain an average number of units equal to 1/(number of SH groups × Tetrakis to HEMA molar ratio). The number of SH groups on each Tetrakis is four.[25] For the Tetrakis to HEMA, molar ratio equals to 0.25%, the average number of units will be equal 100 HEMA molecules. The PHEMA branches are connected together with DMHA cross-linkers. Without Tetrakis hydrogels contain long chains of HEMA molecules coiled together and cross-linked with DMHA.

Swelling and mechanical evaluation of hydrogels

We studied the swelling behavior of hydrogels by measuring the equilibrium water content at swollen state after one

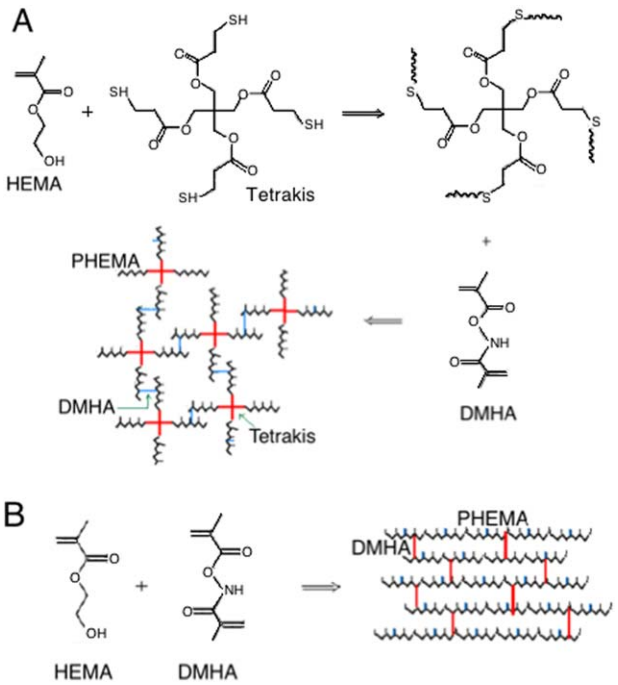

FIGURE 1. Structure of the hydroxyethyl methacrylate (HEMA) hydrogels. A: HEMA cross-linked with N,O-dimethacryloyl hydroxylamine (DMHA) in presence of Tetrakis (HEMA-DMHA-Tetrakis hydrogels). In the presence of Tetrakis, hydrogels present a star structure in which four short PHEMA chains are linked to a Tetrakis molecule. The PHEMA branches are connected together with DMHA cross-linkers. B: HEMA cross-linked with N,O-dimethacryloyl hydroxylamine (DMHA) in the absence of Tetrakis (HEMA-DMHA hydrogels). Without Tetrakis, hydrogels contain long chains of HEMA molecules cross-linked with DMHA.

week of swelling in PBS. We prepared six samples for each of the four hydrogel groups. The equilibrium water content was measured as the ratio of the swelling weight of the hydrogels minus their dried weight, over their dried weight. To dry hydrogels, each sample was first flash frozen in liquid nitrogen and then dried under vacuum (Dynavac FD2, Boronia, Australia) over 3 days. We also measured the initial water content as the ratio of the hydrogels' weight immediately after polymerization minus the dried weight, over the dried weight.

The elastic and viscous properties of the swollen samples were characterized by measuring the elastic modulus and the damping ratio of the hydrogels. For the elastic modulus, a single compressive load up to 10% deformation at the rate of 1% per second was applied with an Instron E3000 linear mechanical testing machine (Instron, Norwood, MA). In this range of deformation, each hydrogel had a linear stress-strain relationship. The elastic modulus was calculated from the slope of the stress−strain curve of each sample. For quantifying the damping ratio, cyclic compression test at 1 Hz and 15% deformation amplitude was applied on samples for 60 s with the Instron machine. The damping ratio was calculated as the ratio of the dissipated work (the area of the hysteresis curve in the force-displacement graph) over the total input work given to the material (the area under the compression curve in the force-displacement graph) during one cyclic deformation.[29] We considered the mean value of damping ratio for the last 10 cycles.

Accelerated degradation study

It has been shown that basic environment expedites the hydrolysis of DMHA.[30] In order to compare the degradation capacity of the produced hydrogels and evaluate the effect of different parameters like the cross-linker ratio, water content, and the presence of the Tetrakis molecules, the mass loss of thin films of hydrogels was measured during 30 days, when they were incubated in highly basic environment. The hydrogel thin films were prepared by cutting the hydrogel samples from each group of S1, S2, S3 and S4 with a Vibratome machine (Leica VT1200S, Muttenz, Switzerland) in 300 μm films (4 mm × 8 mm). Prior to the degradation test, the initial (time zero) dry mass was measured after drying the samples. Then they were moved to multi-plates containers and 3 mL of NaOH 0.01 molar (pH = 12.3) was added to each sample and incubated at 37°C. The NaOH was changed every week. At days 2, 4, 7, 14, and 28, four samples per each hydrogel group were taken, dried, and weighed them with the same procedure as for time zero.

Mechanical properties and weight loss of hydrogels under cyclic loading

Since we developed these HEMA-based hydrogels for load-bearing applications, in order to study the degradation in this situation, we evaluated the mechanical properties and weight loss of hydrogels under cyclic loading. We considered two groups of hydrogels. In the first group, a 1000 cyclic compression load was applied every week while no load was applied in the second group. The cyclic compression consisted of a 15% deformation amplitude at 1 Hz. We used a multi-piston set up, previously designed in our group[31] to be able to apply load simultaneously on multiple samples. In both groups, hydrogels were incubated at 37°C and immersed in 3 mL PBS (pH = 7.4). We changed the PBS every 2 weeks. We monitored the elastic modulus (six samples for each of the four hydrogel groups) and weight loss (four samples for each of the four hydrogel groups) every month during nine months following the same procedures as described under the sections 2.4.

Molecular weight of degradation products

We extracted the degradation products from the PBS media in which the samples were incubated during the degradation study made under the mechanical loading (section 2.6). PBS was collected from each of the four hydrogel groups every month after 4 months of starting the degradation study and was freeze-dried. After freeze-drying, we obtained the degradation products as white powders. The powders were analyzed with Gel Permeation Chromatography (GPC) facility (GPC 50 Agilent, Santa Clara, CA, USA) to quantify the molecular weight of the degradation products.[32,33] The Eluent for GPC consisted of Milli-Q water and 10% MeOH. A conventional calibration was performed with RI (Nicolet Magna-ir

560, Ontario, Canada) and viscometer (Alpha L, Barcelona, Spain). The standards range was 1010 to 278100 Da.

Structure of hydrogels obtained by SEM

The structure of the hydrogels was obtained using a Scanning Electron Microscope (SEM). Cross-sections of the hydrogels were obtained by cracking frozen gels. We prepared a set of swollen new samples of S1, S2, S3, and S4 and a set of samples partially degraded after 6 months under weekly cyclic loading. Samples were dried by freeze-drying, stuck on SEM pads and cross-section of the mid-region of the sample were imaged using an electron microscope (Zeiss Merlin, Oberkochen, Germany) at an accelerating voltage of 0.8 kV and 2.5 Kx magnification, with an aperture of 5 mm and working distance of 6 mm.

Biocompatibility of hydrogels and degradation products

The biocompatibility of the developed hydrogels was evaluated by a direct contact test. Cylindrical hydrogels (three samples for each of the four hydrogel groups) were placed separately in the middle of a 60 mm diameter petri dish and primary human chondrocyte cells isolated and characterized in our group[34] were seeded around the samples (3000 cell/cm^2). Totally, 5 mL of cell culture medium was added to each petri dish. After 1 week, cells were fixed by adding 1 mL methanol to each plate for 30 s following by 1 mL diluted Giemsa solution to color the cells. Microscopy of cells on the surface of the hydrogels and on petri dish was performed in order to visualize fixed, colored cells (ZEISS Axiovert 100, Germany).

In a second test, we evaluated the cytotoxicity of the degradation products. The degradation products were collected at different time points from HEMA-DMHA hydrogels (S1 and S3) or HEMA-DMHA-Tetrakis hydrogels (S2 and S4) obtained in section 2.7. Different concentrations of degradation products (0 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 3 mg/ml) were added in the cell culture medium. The cells' proliferation exposed to the different concentrations was evaluated using a CellTiter assay following the standard manufacturer protocol (CellTiter 96® Aqueous, Promega, Fitchburg, WI) and an absorbance reading at 490 nm with a spectrophotometer (Wallac Victor2, 1420, Turku, Finland). The CellTiter assay was performed every day for 3 days.

Statistical test

We used an ANOVA test to determine if a significant difference (p values < 0.05) is present between each of the four hydrogel groups regarding different parameters like cross-linker, water ratio, presence of Tetrakis, and weekly mechanical load. In conjunction with ANOVA we did a Tukey-Kramer post-hoc test to find means that are significantly different from each other.

RESULTS

Swelling and mechanical evaluation of hydrogels

The equilibrium and initial water contents of the hydrogels are reported in Table II. There was a significant difference between the equilibrium and initial water contents in each group of hydrogels ($p < 0.001$). However, the hydrogels with 1% cross-linker and 65% water ratios (S3 and S4) had higher water content at the equilibrium state compared to the hydrogels with 10% cross-linker and 40% water (S1 and S2) ($p < 0.001$). This confirms that decreasing the amount of cross-linker and increasing the water ratio increase the swelling of the hydrogels. The hydrogels containing Tetrakis (S2 and S4) had slightly higher equilibrium water content compared to the hydrogels without Tetrakis (S1 and S3) ($p < 0.04$). However, the effect of the cross-linker and the water ratios on the swelling of the hydrogels was more significant than the effect of Tetrakis ($p < 0.001$).

TABLE II. Initial and Equilibrium Water Contents

Sample name	Equilibrium Water Content (%)	Initial Water Content (%)
S1	51.51 ± 1.97	41.39 ± 2.03
S2	59.66 ± 8.99	39.57 ± 1.32
S3	133.20 ± 7.32	63.07 ± 4.34
S4	149.36 ± 11.37	59.25 ± 5.45

Significant differences were observed between the equilibrium and the initial water contents in each group ($p < 0.001$). The addition of Tetrakis, a decrease in cross-linker ratio or an increase in water ratio significantly increased the equilibrium water content ($p < 0.04$).

Mechanical characterization of the hydrogels highlights that the elastic modulus highly depends on cross-linker and water ratios. Figure 2(A) shows that the elastic modulus was 10 times lower in hydrogels with less cross-linker and higher water ratios (S1: 2.5 ± 0.21 MPa vs. S3: 0.23 ± 0.019 MPa) ($p < 0.001$). The addition of Tetrakis decreased the elastic modulus of the hydrogels to more than half of its original value (S2: 0.85 ± 0.026 MPa vs S1: 2.5 ± 0.21 MPa and S4: 0.094 ± 0.009 MPa vs S3: 0.23 ± 0.019 MPa) ($p < 0.001$). However, all hydrogels showed high viscous properties [damping ratio greater than 0.6, Figure 2(B)]. While the addition of Tetrakis slightly increased the damping ratio, there was no significant difference between the damping ratios of the different groups ($p > 0.3$).

Accelerated degradation study

Figure 3 shows the weight loss by the hydrogel films incubated in NaOH 0.01 molar (pH = 12.3) over four weeks. The addition of Tetrakis significantly increased the amount of weight loss at each time point ($p < 0.001$). Also, the weight loss by hydrogels with 1% cross-linker and 65% water ratios (S3 and S4) was higher than the other two groups (S1 and S2) ($p < 0.001$). However, the effect of adding Tetrakis was more significant than the effect of cross-linker and water ratios on the weight loss ($p < 0.001$). Hydrogels containing Tetrakis, 1% cross-linker and 65% water (S4) completely degraded in one week.

Mechanical properties and weight loss by hydrogels under cyclic loading

Figure 4 shows the changes in the elastic modulus of all hydrogel groups (S1, S2, S3, and S4) over nine months degradation in PBS, with or without weekly cyclic mechanical

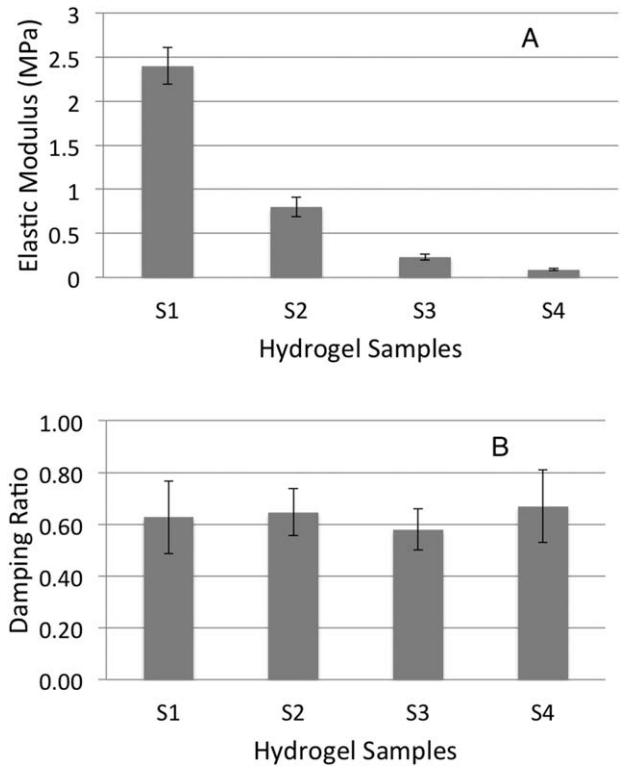

FIGURE 2. A: The elastic modulus of the four groups of hydrogels. Hydrogels with 1% cross-linker ratio and 65% water content (S3 and S4) had smaller elastic modulus than the ones with 10% cross-linker ratio and 40% water content (S1 and S2) ($p < 0.001$). Hydrogels containing Tetrakis (S2 and S4) had lower elastic modulus compared to those without Tetrakis (S1 and S3) ($p < 0.001$). B: The damping ratios of the four groups of hydrogels. There was no significant difference between the damping ratios of the different groups ($p > 0.3$).

loading. The elastic modulus decreased over time, in some cases, by [mt]90%. For each group, applying a mechanical load induced a significant decrease in the elastic modulus value at each time point ($p < 0.001$). However, changing the cross-linker and water ratios and adding Tetrakis had no significant influence on the final percentage of elastic modulus loss after nine months ($p > 0.1$). Furthermore, we observed that the rate of loss of elastic modulus (the slope of the curve) was higher during the first two months of degradation for all hydrogels.

In Figure 5, the weight loss by all hydrogel groups during the nine months of degradation, with or without weekly cyclic loading, is reported. For hydrogels S1, S2, and S4, applying mechanical load resulted to a significant increase in the amount of weight loss at each time point ($p < 0.001$). However for the group S3, this effect was observed only after five months. The addition of Tetrakis significantly increased the amount of weight loss in all hydrogel groups over the entire degradation period ($p < 0.001$). A correlation was found between applying mechanical loading and adding Tetrakis ($p < 0.01$), suggesting that having both parameters highly increases the degradation. However, from our results, the effect of cross-linker and water ratios on the amount of weight loss was not clear. While for hydrogels containing Tetrakis the amount of weight loss after nine months was greater for hydrogels with 1% cross-linker ratio and 65% water content (S4) compared to hydrogels with 10% cross-linker ratio and 40% water content (S2), we observed an opposite phenomenon in hydrogels without Tetrakis (S3 and S1). Furthermore, we observed that for hydrogels containing Tetrakis the slope of the weight loss curves increased in the later months of degradation, while for the hydrogels without Tetrakis the slope of the curves decreased. This suggests that the degradation of hydrogels containing Tetrakis increases in the later months, while this is less likely to happen in hydrogels without Tetrakis.

Molecular weight of degradation products

The results of GPC test showed that there was no significant difference in the molecular weight of degradation products between the hydrogels with 10% cross-linker (S1 and S2) and those with 1% cross-linker ratio (S3 and S4) ($p > 0.5$). Therefore, we considered highly cross-linked and little cross-linked groups together and we only separated groups by whether or not they contained Tetrakis. All the samples without Tetrakis had a peak in the GPC graph ranging between 7000 and 9000 kDa (7668 ± 816 kDa). However two samples had also a second peak, one at 4683 kDa, and the other at 2966 kDa. For samples with Tetrakis we detected two peaks for all samples, one peak between 4500 and 7500 kDa (6096 ± 1544 kDa), and another peak between 2500 and 3200 kDa (2820 ± 351 kDa).

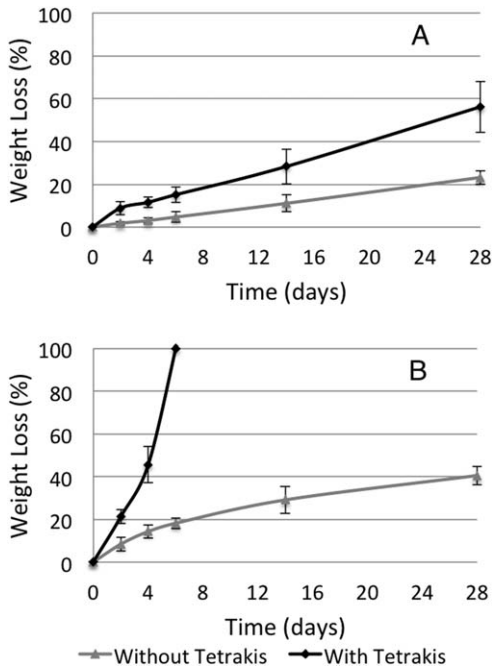

FIGURE 3. The amount of weight loss of the hydrogels in an accelerated degradation study. A: Hydrogels with 10% cross-linker ratio and 40% water content (S1 and S2), B: hydrogels with 1% cross-linker ratio and 65% water content (S3 and S4). The addition of Tetrakis significantly increased the amount of weight loss at each time point ($p < 0.001$). Hydrogels with a lower cross-linker ratio and a higher water content (S3 and S4) lost weight faster than the other groups (S1 and S2) ($p < 0.001$). Hydrogels in group S4 completely degraded after 1 week.

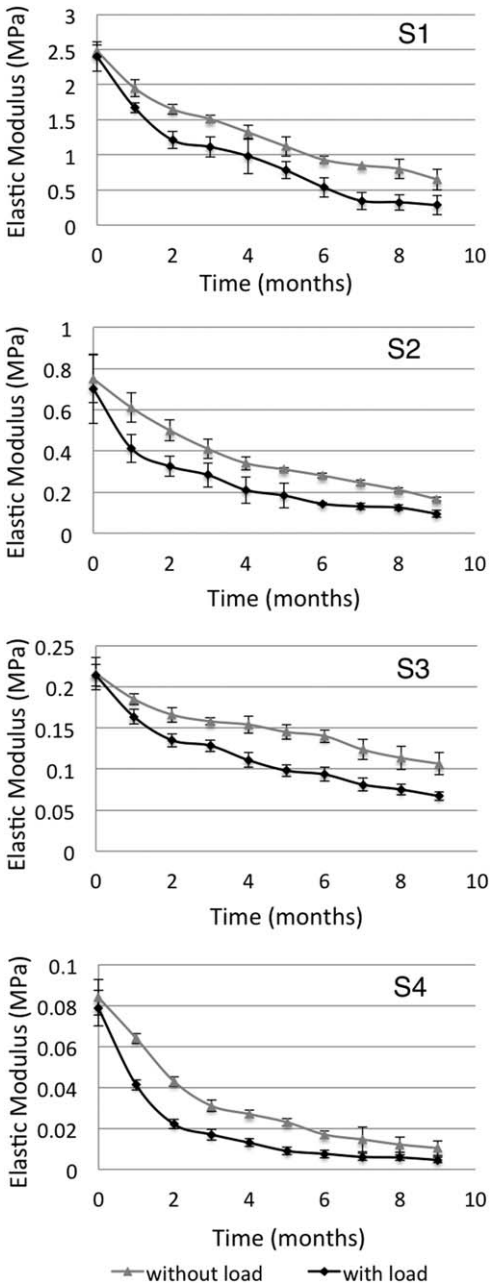

FIGURE 4. Evolution of the elastic modulus of the hydrogels (S1, S2, S3, and S4) during degradation in PBS, with and without applying weekly cyclic loading. For each group, applying a mechanical load induced a decrease in the elastic modulus value at each time point ($p < 0.001$). Changing the cross-linker and water ratios and adding Tetrakis had no significant influence on the final percentage of elastic modulus loss after nine months ($p > 0.1$). The final percentage of elastic modulus loss after nine months was S1: $88.23 \pm 7.54\%$, S2: $86.57 \pm 5.35\%$, S3: $78.96 \pm 8.32\%$, and S4: $94.15 \pm 8.76\%$.

Structure of hydrogels obtained by SEM

The morphology and porosity of the hydrogels were examined by SEM (Figure 6). We could not observe the porous structure of highly cross-linked hydrogels (S1 and S2) since the structure was too dense to be observed with SEM at the voltage of 0.8 kV and increasing the voltage ruined the samples. In contrast, we were able to see the porous structure of hydrogels with 1% cross-linker and 65% water containing or not Tetrakis (S3 and S4). As Figure 6 shows, in the presence of Tetrakis, the hydrogel has larger and rounder pores, which seem to be less interconnected to each other [Figure 6(A,B)]. We also examined these hydrogels after six months of degradation under weekly cyclic load. The

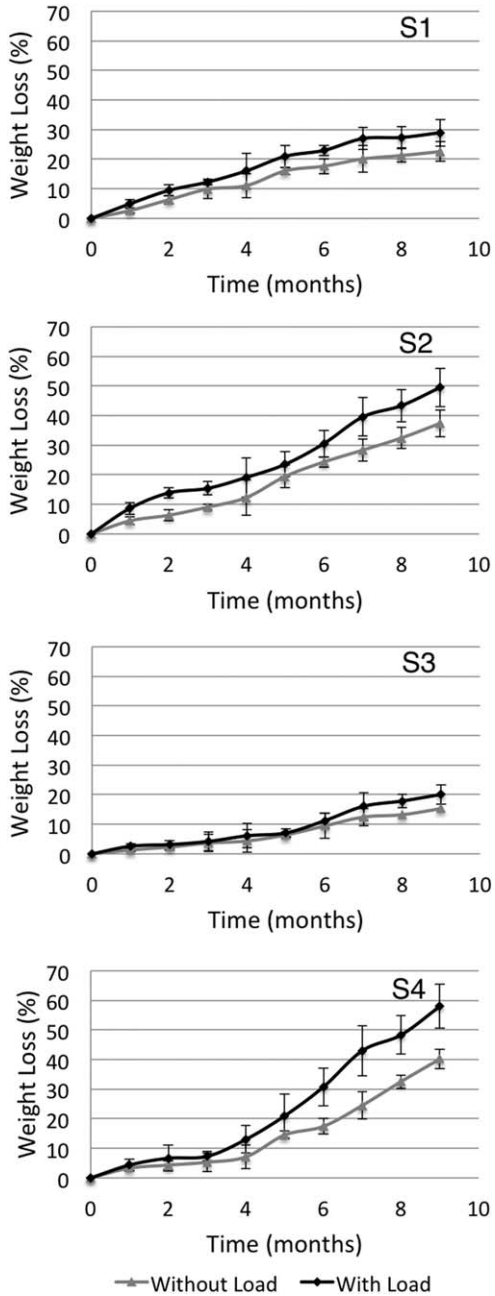

FIGURE 5. The amount of weight loss of the hydrogels (S1, S2, S3, and S4) during degradation in PBS, with and without applying weekly cyclic loading. For each group, applying a mechanical load increased the amount of weight loss at each time point ($p < 0.001$) except the first 5 months of the group S3. Also, the groups with Tetrakis (S2 and S4) showed a greater weight loss compared to the groups without Tetrakis (S1 and S3) ($p < 0.001$). Changing the cross-linker and water ratios had no significant influence on the amount of weight loss ($p > 0.5$).

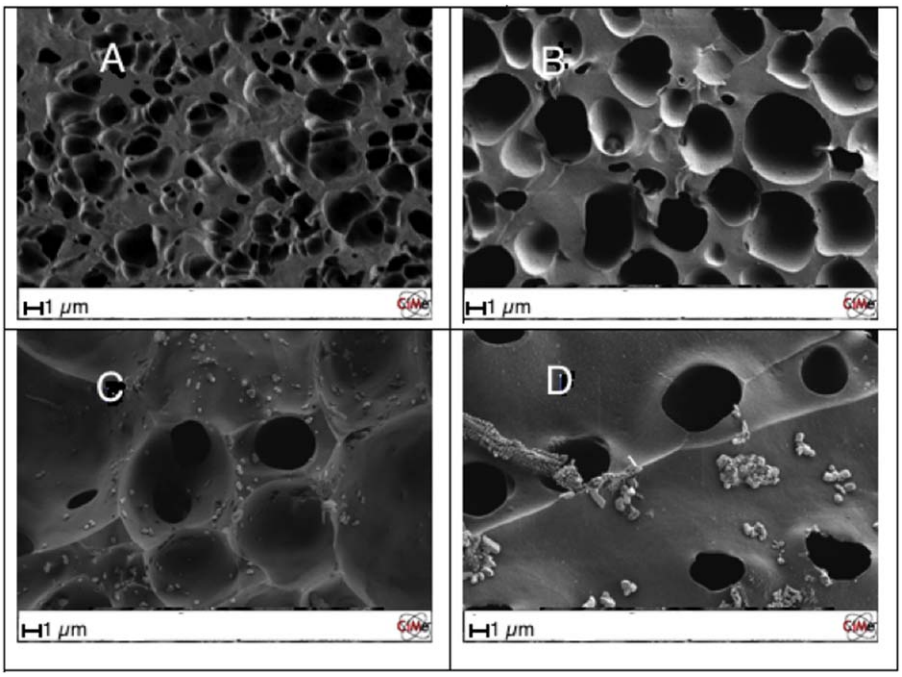

FIGURE 6. Scanning Electron Microscopy pictures of the hydrogels. A: S3 at zero time point. B: S4 at zero time point. C: S3 after six months of degradation under cyclic loading. D: S4 after six months of degradation under cyclic loading.

structure of the partly degraded hydrogels was highly porous and interestingly we could visually detect the particles of the degradation product on the wall of the pores [Figure 6(C,D)].

Biocompatibility of hydrogels and degradation products

The morphology and proliferation of the cells in contact with the hydrogel samples were similar to those seeded on standard cell culture plates. Figure 7 shows a typical Giemsa staining for hydrogel S2. The microscopic picture of the colored cells demonstrated that their density and shape in contact with the hydrogel (Zone 1) were similar to the areas far from the hydrogel (Zone 2) on the cell culture plate.

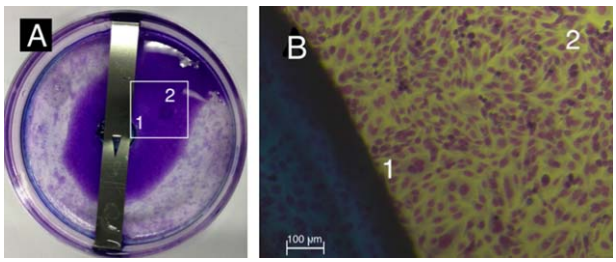

FIGURE 7. Giemsa staining of the sample S2 to visualize cells' distribution around the hydrogel. A: an overview of the area of interest (white rectangle). B: microscopic picture of the area of interest. In each image, the label (1) corresponds to the cells near the hydrogel and the label (2) corresponds to the cells on cell culture plate far from the hydrogel.

The evaluation of the cytotoxicity of the degradation products over three days confirmed that these products did not affect the cells proliferation. In Figure 8, we can observe that, at each day, there was no significant difference between the absorbance signal of cells cultured in mediums containing different concentrations of degradation products and the control group, which contains no degradation product ($p > 0.25$).

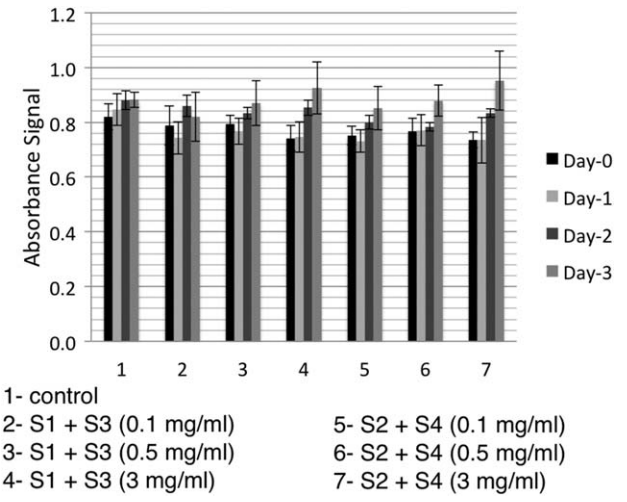

FIGURE 8. Spectrophotometry absorbance signal for CellTiter test, when different concentrations of degradation products from HEMA-DMHA hydrogels (S1 and S3) or HEMA-DMHA-Tetrakis hydrogels (S2 and S4) were exposed to cells for 3 days. No significant difference could be found between the mean values of the absorbance signal of each group containing the degradation products and the mean values of the control groups at each day ($p > 0.25$).

DISCUSSION

We developed a biodegradable photo-crosslinked hydrogel based on HEMA with high mechanical properties for load-bearing applications. Our original formulation consisted of HEMA cross-linked with a biodegradable N, O-dimethacryloyl hydroxylamine (DMHA) molecule and a tetra-functional chain transfer agent pentaerythritol tetrakis in the backbone of the hydrogel. We studied the degradation under cyclic mechanical loading, which provides a more realistic environment to evaluate the degradation of hydrogels in load-bearing conditions. Mechanical loading can increase water circulation inside the porous structure of hydrogels, which favors hydrolysis and helps to remove the degradation products.

The obtained HEMA-DMHA-Tetrakis hydrogels degraded in low molecular weight products over a prolonged time period. With respect to its mechanical properties, the elastic modulus of the hydrogels decreased at the initial stage of the degradation process. Since the elastic modulus of the hydrogels is proportional to their cross-linker ratio,[35] we concluded that the decrease in the elastic modulus during the early months of degradation was related to the hydrolysis of the DMHA cross-linker. The increase of the weight loss rate after several months for the hydrogels containing Tetrakis confirmed that in these hydrogels, their backbone started to break apart during a second stage of degradation. At this stage, the hydrogels began to lose their mechanical integrity. If the hydrogels did not contain any Tetrakis, the degradation could not be completed as in this situation, after the hydrolysis of the cross-linker, the structure of the hydrogels consisted of long nondegradable PHEMA chains which could coil and keep their integrity via some hydrophobic interactions.[36] The presence of Tetrakis allowed the long PHEMA chains to be broken into shorter-length molecules presenting a star structure. This effect has already been described by Chiellini et al.[25] Having low molecular weight degradation products is one of the most important aspects when developing biodegradable hydrogels. Since the degradation products of our hydrogels were <10 kDa, they could be cleared out of body.[6,27]

It should be noticed however that we did not observe a complete degradation of the hydrogels over the nine months degradation study. Slow degradation rate of our hydrogels and their high mechanical properties could anyway be desirable for scaffolding in load-bearing applications. Long-term degradation mirrors the rate of tissue development in load-bearing tissues like cartilage and nucleus pulposus where the healing process is slow.[37–39] In parallel, the high damping properties observed in the develop hydrogels provided load and crack resistance under the loading conditions of such tissues.[12,18]

It has been already shown that HEMA-based hydrogels can partially degrade when they are cross-linked to some natural polysaccharides molecules such as chitosan,[22] dextran-based molecules,[7,40] hyaluronic acid and poly(lactic acid) PLA.[35] Due to the complexity of the functionalization of most of these molecules, their high molecular weight, and their mechanism of cross-linking, resulting hydrogels usually have very poor mechanical properties and cannot be used for load-bearing applications. Furthermore, in most cases, the high molecular weight of the resulting degradation products has been reported to be an issue. For example, Atzet et al. has developed a biodegradable PHEMA gels cross-linked with polycaprolactone (PCL) which presented high and tunable mechanical properties, but also had degradation products with high molecular weight.[6] The degradation properties of DMHA, which we used, have been already characterized in the literature.[28,30,41] We chose this molecule because it is a hydrolysable short-length molecule similar in size to HEMA monomers. We have previously shown that using cross-linkers similar in size to the HEMA monomers increased the viscous properties of the HEMA-based hydrogels, which favored then their crack resistance.[18] DMHA undergoes base catalyzed hydrolysis at pH values above 5, thus it is a useful cross-linker which can be cleaved at a physiological pH of 7.4.[28,30] Huang et al. reported partial weight and stiffness loss in hydrogels made of PHEMA cross-linked by DMHA.[26] Indeed, these authors showed that the weight loss reached a constant value (20%) after several months. Our results presented the same degradation profile for HEMA-DMHA hydrogels containing no Tetrakis. As mentioned before, this is due to the long interconnected PHEMA chains in the backbone of hydrogels. Based on the results of this study, using Tetrakis in the backbone of hydrogels solved this issue. Chiellini et al. has already used Tetrakis molecules to increase the degradation properties of PHEMA hydrogels.[25] However, in their study, they used poly(ethylene glycol di-methacrylate) (PEGDM) as a cross-linker, which is nondegradable. To the best of our knowledge, no other study has tried to use the advantage of Tetrakis molecules in PHEMA hydrogels.

There are some limitations in our study. We evaluated the degradation properties of two HEMA-DMHA-Tetrakis hydrogels as a model system. The first hydrogel was obtained with a low cross-linker ratio and high water content, which can mimic soft-tissues like nucleus pulposus. The second one was made with a high cross-linker ratio and low water content, with resulting mechanical properties similar to articular cartilage. We did not study the effects of these two parameters (cross-linker ratio and water content) separately on the degradation and the mechanical properties of the hydrogels. The effects of these two parameters on the mechanical properties of HEMA-based hydrogels have already been studied for similar geometry cross-linkers like ethylene glycol dimethacrylate (EGDMA).[3,16–18] Another limitation is related to the GPC method used to measure the molecular weight of the degradation products. This method can only identify the different molecular weights of material composing the hydrogels, but it cannot bring any information on their respective distribution. Nevertheless, the presence of peaks at lower molecular weights in the GPC signal of hydrogels containing Tetrakis strongly suggests that using this molecule decreases the molecular weight of the degradation products. Furthermore, we were still able to show that the molecular weight of all degradation products even from hydrogels without Tetrakis was smaller than the required limit for being cleaned out of the body.

CONCLUSIONS

In conclusion, the new formulation of the HEMA-DMHA-Tetrakis hydrogels allows us to obtain a promising slow degrading biomaterial presenting low molecular weight degradation products, along with tunable mechanical properties. With such properties, the obtained hydrogels could overcome the existing limitations in the application of HEMA-based hydrogels in tissue engineering and drug delivery, especially for load-bearing applications.

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